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Larvicidal activities of some plants essential oils of the Cameroonian flora against *Anopheles gambiae* S.L., major vector of malaria in the city of Douala

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Abstract
The present study aims to investigate the larvicidal activity of essential oils of the rhizomes of *Zingiber officinalis*, the leaves of *Tithonia diversifolia* and *Bidens pilosa* on the mature and immature larvae of *Anopheles gambiae* s.l. The chemical composition of the essential oils was determined by Gas Chromatography (GC) and Gas Chromatography and Mass Spectrometry (GC/MS) coupling. The larvicidal activities of essential oils against *An. gambiae* s.l. was monitored in the laboratory using the world health organisation protocol. Chemical analysis has shown that all these oils are dominated by monoterpene α-pinene was the major compound found in the essential oils of the leaves of *T. diversifolia* (69.53%) and *B. pilosa* (64.43%); while geranial was the majority compound found in the essential oil of *Z. officinalis* (35.40%). Larvicidal tests showed that the essential oil of the leaves of *T. diversifolia* was the most effective (LC50 = 70.18 ppm and 92.49 ppm respectively for larvae of immature and mature stages after 24 hours of exposure). From the above, the essential oil of the leaves of *T. diversifolia* can be recommended for the development of natural biocides against malaria vectors.

Keywords: *Zingiber officinalis*, *Tithonia diversifolia*, *Bidens pilosa*, Essential oils, Larvicidal activity, Douala

Introduction
Malaria is one of the vector-borne diseases that affect a large part of the world's population. In Africa, nearly 93% of people are affected by the disease. Pregnant women and children under 5 years are the most exposed segments of the population [1]. In Cameroon, malaria is a major cause of morbidity. For several decades, Cameroon has been deploying a lot of measures to eradicate this parasitosis which is a brake on its development and the well-being of the populations. These include the massive and free distribution of long-lasting impregnated mosquito nets to all households and the free treatment of simple malaria of children under 5 years.
Notwithstanding these various measures implemented to control this disease, the results remain mixed. According to the Minister of Public Health, Cameroon is one of the 11 countries in the world most affected by this disease. The reported data show more than 3, 150, 784 cases of contamination for 3,863 deaths. In addition, malaria represents 30% of the reason for consultation, a figure that is increasing compared to 2019 [2]. This shows a rise in the incidence of malaria in our country, despite the efforts made so far to combat this disease. Multiple causes are mentioned to justify such a situation, ranging from the limited application of existing means of prevention and treatment to vector resistance to the usual insecticides. Several studies conducted in this context show resistance of vector populations to synthetic insecticides currently used in vector control programs [3]. To date, the study of insecticidal susceptibility has been carried out in the populations of *An. gambiae* S.L., *An. funestus* and *An. Moucheti*. With the exception of organophosphates, the populations of *An. gambiae* are resistant to most molecules used in vector control [4].

This resistance is highlighted in *An. arabiensis*, *An. coluzzii* and *An. gambiae*. In the populations of *An. gambiae* and *An. Coluzzii*, resistance is caused by the presence of kdr gene alleles and/or overexpression of detoxification enzymes [4]. With this in mind, it becomes urgent to look for new low-toxic molecules whose effectiveness is proven in order to preserve nature and non-target populations. Essential oils of natural plants have significant advantages over synthetic molecules, as they still have some effectiveness against several organisms and are less aggressive for the environment. For centuries, plants have been constantly used to fight against many organisms. In the different regions of Cameroon, a various range of plants is used by populations to control the aggression of vectors against humans, animals and other plants. Various works carried out on the national territory highlight the insecticidal potential of several plants from the local flora [5, 6, 7, 8, 9]. These plants for the most part have essential oils, are rich in functional groups and have properties to diffuse easily through cell membranes. A total of three plants from the local flora caught our attention as part of this study. They are *Bidens pilosa; Tithonia diversifolia* and *Zingiber officinalis*. These plants have been the subject of several studies that show their pesticidal effect on a variety of organisms including insect pests [10, 11]. It is with this observation that we proposed to evaluate the insecticidal activities of the essential oils of the various plants mentioned above on the larvae of *Anopheles gambiae* s.l. in the city of Douala.

Materials and Methods

Study area

The city of Douala, the economic capital of Cameroon, is located in the equatorial zone and at the bottom of the Gulf of Guinea, along the estuary of the Wouri River. It is located on the banks of the Wouri River 30 km from the Atlantic Ocean. It is located between 4° and 4°10' north latitude and between 9°35' and 9°80' east longitude. Douala is characterized by a plain relief, consisting of small hills not exceeding 60m in height and notched by a dense and winding network of streams and streams, tributaries of coastal rivers. The Wouri, the only river crossing the city of Douala in its northwestern part, flows in a NE-SO direction and is fed by a few rivers crossing the northern and western districts. The population is cosmopolitan and lives for the most part from trade. The poor management of usual waste is often at the origin of cottages conducive to the development of mosquitoes.

Plants collection and essential oils extraction

The plants were selected on the basis of data collected in the literature and on the basis of the uses made by the local populations. These plants were collected between January and May 2020 in different localities of the city of Douala and then identified at the Laboratory of Biological Plant of the University of Douala. The rhizomes of *Zingiber Officinalis*, the leaves of *Tithonia diversifolia* and *Bidens pilosa* were collected, washed with spring water and then subjected to extraction by steam drive. The oil collected following distillation by decantation, was filtered on anhydrous sodium sulphate column and then stored at 4 ° C, inside a refrigerator, in hermetically sealed and dark glass vials.

Chemical composition analysis of essential oils

The chemical analysis of the essential oils was carried out using a Variant CP-3380 chromatograph equipped with a flame ionization detector and a capillary column (length 30 m, internal diameter 0.25 mm) with a non-polar stationary phase of the methylsilicone type (DB-1, film thickness 0.25 μ). Nitrogen is used as a carrier gas with a flow rate of 0.8 ml.min-1. The temperature of the injector is 220 °C; the detector is set to 250 °C. The furnace was programmed from 50 °C to 200 °C with a temperature gradient of 5 °C.min-1. The retention indices of the different constituents were calculated in relation to the retention times of a series of n-alkanes and their relative percentages calculated by electronic integration considering that their response factors are all equal to 1. The gas chromatography – mass spectrometry coupling was carried out using Hewlett Packard HP 5970 A brand apparatus, equipped with a non-polar capillary column (30 m x 0.25 mm) made of HP1 fused silica (film thickness 0.25 μ) and a quadrupole detector (ionization energy 70 eV). The temperature of the injector was 220 °C and that of the interface area was 210 °C. Injection in split mode (1/100) of 1 μl of a 10% essential oil solution in dichloromethane. The furnace temperature was programmed from 70 °C to 200 °C with a gradient of 10 °C.min-1. The carrier gas was helium with a flow rate of 0.6 ml.min-1. The acquisition was performed in scan mode [35-300 amu] at 2.96 scan.sec-1. The identification of the constituents of essential oils was made on the basis of their retention indices and mass spectra in comparison with the data of the Literature and Adams [12].

Collection, breeding of Anopheles and biological tests

All potential gites were visited in our study area. The cottages containing the larvae were systematically emptied of their contents and poured into plastic basins (30x10x20cm). The larvae were collected by sieving and introduced into plastic jars of 500ml capacity and then raised until the adults were obtained. The larvae from the F1 generation were fed with the protein- and mineral-enriched powder, Tetra baby fish food, diluted in small amounts of water and introduced into the breeding boxes. After 24 hours, the larvae were taken using a pastoral pipette and divided into batches of 20 individuals in bowls 8cm in diameter and following their immature (L1, L2) or mature (L3, L4) stage. Each of the bowls contained 99ml of well water in which 1ml of diluted test solution was added. This is based on the protocol of the World Health Organization (1985). Preliminary experiments made it possible to select a range of concentrations for the tests. As a result, essential oil stock solutions from each sample were prepared in 90° ethanol. From these daughter dilutions were carried out to obtain final experimental concentrations of 75, 100 and 150ppm. Three repetitions were performed for each of the dilutions. Two control cups were also made under the same conditions as the test bowls. The negative control contained only ethanol (in the same proportions as for the tests, i.e. 1%) without any trace of essential oil. The counting of the larvae was carried out every 5 minutes for 1 hour; then every hour for 10 hours and finally mortality was measured after 24 hours of exposure to volatile extracts solubilized in water.

Results and Discussion

Extraction yield: The extraction yields of essential oils from plant leaves and rhizomes are recorded in Table 1. The rhizomes of *Z. officinalis* (1.36%) have a higher essential oil content than the leaves of *T. diversifolia* (0.1%) and those of *B. pilosa* (0.042%). Many studies argue that plant essential oil yields vary depending on the plant organ extracted, the method of extraction, climatic conditions, the geographical location of the harvest site, the harvest period, and the pathophysiological state of the plant at harvest time [13, 14, 6]

Table 1: Extraction efficiency of essential oils from the leaves of *T. diversifolia, B. pilosa* and the rhizomes of *Z. officinalis*

Plants			Harvest			Essentials oils		Yield
Family	**Species**	**Organs**	**Mass (g)**	**Date**	**Place**	**Color**	**Mass (g)**	**(%)**
Zingiberaceae	*Zingiber officinalis*	Roots	500	15/04/21	Douala	yellow	6,8	1,36
Asteraceae	*Bidens pilosa*	Leaves	7000	15/04/21	Douala	dark	3	0.042
Asteraceae	*Tithonia diversifolia*	Leaves	12000	15/04/21	Douala	Yellow	12	0.1

Chemical composition of essential oils: The results of analysis of the different essential oils are recorded in Table 2 below. It appears from this table that oxygenated monoterpenes are the compounds most represented in the essential oil of *Z. officinalis*. While hydrocarbon monoterpenes (29.08% and 24.41%) are the compound most represented in the essential oils of *T. diversifolia* and *B. pilosa* respectively. *Z. officinalis* essential oil has a high content of oxygenated monoterpenes (30.66%) with geranial (13.60%) as the major compound. This result is contradictory to those of previous work carried out on the essential oil of this plant species native to Benin and Vietnam. According to Sessou, *Z. officinalis* essential oil native to these countries was rich in Zingiberene [15, 16]. In addition, the essential oil of *T. diversifolia* has a high content of hydrocarbon monoterpenes (29.08%) with α-pinene (18.97%) as the major compound. The essential oils of *T. diversifolia* leaves collected in Vietnam (30.7%) and Nigeria (34.42%) have a high proportion of α-pinene molecule [17, 18]. These results are similar to those obtained by Oludare [19]. According to the latter, α-pinene is the major constituent of the essential oil of *T. diversifolia*. In view of these different results, α-pinene represents the major component and the characteristic molecule of the essential oil of *T. diversifolia*. As for the essential oil of B. pilosa predominantly monoterpene hydrocarbon (24.41%), α-Pinene is the major constituent (16.86%). This result contrasts with those of the work carried out in Nigeria, which reports that the essential oil of *Bidens pilosa* leaves collected in Nigeria (82.3%) has a high content of sesquiterpene compounds, of which carophyllene oxide (37.3%) represents the major compound [20]. Our result corroborates that of the work carried out by Goudoum, in the northern region of Cameroon and whose conclusions show that α-Pinene is the major compound found in the essential oil of *Bidens pilosa* [21]

Table 2: Chemical composition of essential oils of *Z. officinalis* ; *T. diversifolia* et *B. pilosa.*

Chemical compounds	Percentages			
	R.T	***Z. officinalis***	***T. diversifolia***	***B. pilosa***
Ketones		11,59	-	-
2- Octen-4-one	8.354	11.12	-	-
n- Nonane-2-one	6.037	0.47	-	-
Monoterpenes		41,88	30,03	27,14
MTH		11,22	29,08	24,41
α- Pinene	3.743		2.15	18.97
Camphene	3.964		6.87	1.18
Myrcene	4.359		0.27	4.39
Sabinene	4.503		1.05	0.24
β- Pinene	4.365	-	1.98	-
α- Phellandrene	4.744	0.41	1.18	-
α- Terpinene	4.936	-	0.22	1.41
P- Cymene	5.068	-	0.92	-
α- Terpinolene	6.037	0.47	-	3.31
Δ-3- Carene	3.636	-	-	1.85
MTO		30,66	0,95	2,73
Linalool	6.176	1.70	-	0.45
α- Terpineol	7.619	1.86	0.30	-
Citronellol	8.119	1.61	-	-
Terpinen-4-ol	7.424	-	0.22	2.28
Bornyl acetate	9.004	-	0.43	-
Geranial	8.781	13.60	-	-
Neral	8.354	11.12	-	-
Eugenol	9.988	0.77	-	-
Sesquiterpenes		10,29	9,14	2,8
STH		9,03	8,11	2,06
β- Elemene	10.481	0.41	-	0.20
α- Copaene	10.278	0.43	-	-
α- Humulene	11.337	-	0.54	0.31
α- Cubebene	10.278	0.43	-	-
Δ- Cadinene	12.168	-	0.39	
β- Cubebene	13.600	-	-	0.28
Trans-caryophyllene	10.897	-	2.86	-

α- Farnesene	11.903	4.22	-	-
Germacrene D	11.678	1.00	1.46	-
Valencene	11.427	0.17	-	-
B- Bisabolene	11.952	2.37	-	-
α- Caryophyllene	10.896	-	-	1.27
β- Caryophyllene	10.897	-	2.86	-
STO		1,26	1,03	0,75
Sesquisabinene hydrate	13.422	0.83	-	-
(E)- Nerolidol	12.589	0.43	0.47	0.36
Methyleugenol	9.992	-	-	0.16
Caryophyllene oxide	12.960	-	0.56	0.23

Biological tests : The results of the various tests show that the essential oils from the roots of *Zingiber officinalis* and those from the leaves of *Bidens pilosa*, *Tithonia diversifolia*, have proven insecticidal properties on larvae of immature and mature stages of *Anopheles gambiae* s.l.. This larvicidal activity is expressed differently from one essential oil to another and according to the concentrations (Table 2). LC50 and LC95 values, determined from Henry's simplified table that transforms cumulative mortality rates into probits, were used to classify these essential oils according to their degree of toxicity on larvae of *An. gambiae* s.l.

Table 3: Lethal concentrations of essential oils (ppm) of *Z. officinalis* roots and leaves of *B. pilosa*, *T. diversifolia* capable of causing 50% (LC50) and 95% (LC95) mortality of larvae of mature and immature stages of *An. gambiae* S.L. after 24 hours of exposure

	Larval stage			
Species	**Immature**		**Mature**	
	LC_{50}	**LC_{95}**	**LC_{50}**	**LC_{95}**
Z. officinalis	89,97	115,48	80,70	103,40
B. pilosa	86,20	110,80	84,43	107,62
T. diversifolia	94,05	94,05	70,57	90,93

Thus the essential oil of *T. diversifolia* was the most effective on larvae of immature stages followed by those of *B. pilosa* and *Z. officinalis*. On the other hand, on larvae of mature stages, the essential oil of *T. diversifolia* was the most effective followed by those of *Z. offinalis* and *B. pilosa* (Table 3). This toxicity of the essential oils of *T. diversifolia* and *B. pilosa* would be due to their high content of α-pinene. The insecticidal activity of this molecule was demonstrated by Ojimelukwe[22] during their work to evaluate the insecticidal activity of α-pinene vis-à-vis *Tribolium confusum*. Multiple studies highlight the important role that so-called minority compound could play in the toxicity of essential oils. Even more hypotheses have been made about the synergistic action of α-pinene and myrcene on the increase in the toxicity of an oil. Thus the high toxicity of the essential oil of *T. diversifolia* would be due to the combined action of the α-pinene and myrcene contained in this oil. As for mortality rates, they differ according to the two larval stages. In addition, the present study shows a high sensitivity of immature larvae compared to mature larvae when they are exposed to different essential oils. Indeed, after 5 minutes of exposure to 150ppm of the essential oils of *Z. officinalis* and *T. diversifolia*, more than 50% of mortality of larvae of immature stages were recorded against less than 5% of mortality of larvae of mature stages. This result can be explained by the natural differences between the two stages because immature larvae have a young and growing integument that could let essential oils diffuse easily unlike mature larvae that have a better constituted integument (Table 4 and 5).

Table 4: Mortality rate according to the duration of exposure of larvae of immature stages of *An. gambiae* in different concentrations of the essential oils *Z. officinalis, T. diversifolia* and *B. pilosa.*

	Times						
Essentials oils concentration(ppm)	**5min**	**1/2H**	**1H**	**10H**	**24H**	**H**	**P**
Z. officinalis							
75	11,67	53,33	91,67	93,33	98,33	4,020	0,43
100	8,33	96,67	100,00	100,00	100,00	4,020	0,43
150	50,00	100,00	100,00	100,00	100,00	4,020	0,43
H	4,020	4,020	4,020	4,020	4,020	-	-
P	0,43	0,43	0,43	0,43	0,43	-	-
B. pilosa							
75	0,00	21,67	56,67	86,67	91,67	4,020	0,43
100	21,67	81,67	85,00	85,00	96,67	4,020	0,43
150	31,67	98,33	100,00	100,00	100,00	4,020	0,43
H	4,020	4,020	4,020	4,020	4,020	-	-
P	0,43	0,43	0,43	0,43	0,43	-	-
T. diversifolia							
75	11,67	91,67	100,00	100,00	100,00	4,020	0,43
100	45,00	91,67	100,00	100,00	100,00	4,020	0,43
150	73,33	100,00	100,00	100,00	100,00	4,020	0,43
H	0,02	0,02	0,02	0,02	0,02	-	-
P	1,02	1,02	1,02	1,02	1,02	-	-

Table 5: Mortality rate according to the duration of exposure of mature larvae of *An. Gambiae* in different concentrations of *Z. officinalis, T. diversifolia* and *B.pilosa* essentials oils.

Essentials oils Concentration (ppm)	Times 5min	1/2H	1H	10H	24H	H	P
Z. officinalis							
75	0,00	3,33	13,33	18,33	5,00	4,020	0,43
100	0,00	26,67	56,67	65,00	63,33	4,020	0,43
150	1,67	56,67	73,33	73,33	93,33	4,020	0,43
H	0,02	0,02	0,02	0,02	0,02	-	-
P	1,02	1,02	1,02	1,02	1,02	-	-
B. pilosa							
75	0,00	0,00	5,00	5,00	5,00	4,020	0,43
100	0,00	10,00	15,00	16,67	25,00	4,020	0,43
150	1,67	55,00	75,00	88,33	86,67	4,020	0,43
H	0,02	0,02	0,02	0,02	0,02	-	-
P	1,02	1,02	1,02	1,02	1,02	-	-
T. diversifolia							
75	0,00	1,67	6,67	6,67	0,00	4,020	0,43
100	0,00	38,33	66,67	71,67	61,67	4,020	0,43
150	5,00	65,00	83,33	88,33	93,33	4,020	0,43
H	0,02	0,02	0,02	0,02	0,02	-	-
P	1,02	1,02	1,02	1,02	1,02	-	-

Conclusion

Based on this result, it would be wise, in cases of implementation of antilarval programmes, to target immature larvae much more. This study shows that the essential oil of *Tithonia diversifolia* has remarkable larvicidal properties against *Anopheles gambiae* compared to other oils.

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